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Properties of deproteinized bone for reparation of big segmental defect in long bone

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Objective: To explore suitable scaffold material for big segmental long bone defect by studying the properties of the prepared deproteinized bone.

Methods: Cancellated bone were made as 30 mm × 3 mm × 3 mm bone blocks from inferior extremity of pig femur along bone trabecula. The deproteinized bone was prepared with an improved method. Their morphological features, components, cell compatibility, mechanical and immunological properties were investigated respectively.

Results: Deproteinized bone maintained natural reticular pore system. The main organic material is collagen Ⅰ and inorganic composition is hydroxyapatite. It has good mechanical properties, cell adhesion rate and histocompatibility.

Conlusion: This deproteinized bone can be applicable as scaffold for reparation of big segmental defect in long bone.

Key words: ***Deproteinized bone; Long bone; Bone defect***

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Scaffold is an important material for bone tissue engineering. For its extensive source and special biological characteristics, deproteinized bone is the main concern for many researches. There are many methods for its preparation.[1]

Domestic and foreign scholars have conducted many studies on the optimization of deproteinized bone preparation to obtain the ideal scaffold material in bone tissue engineering, which can meet the the needs of bone defect treatment. However, so far, heterogeneous deproteinized bone has not been widely used in clinic. In this research, we improved the preparation process and evaluated its comprehensive performance in order to provide references to further research and clinical application.

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METHODS

Materials

The materials and instruments used in this study were as follows: fresh commercial adult pig femur, green goat (Animal Center of Third Military Medical University, China), Co^{60} irradiation machine (the Radiation Center in Southwest Hospital Affiliated to Third Military Medical University, China), NaN_3, NaOH, methanol, chloroform, H_20_2, ethylenediamine, Triton X-100 (Sigma, USA), scanning electron microscopy (Amrar, USA), X-ray diffraction analyzer and X-ray scattering analyzer (D/MAX-rA, Japan), infrared spectrometer (Perkin Elmer Corporation) and RGT-5A omnipotent biomechanical testing instrument (Shenzhen Repaglinide Co., China).

Preparation of deproteinized bone

The heterogeneous deproteinized bone were prepared with a modified method.[2-3] After the removal of soft tissue, cancellated bone of fresh pig femur were made as 30 mm × 3 mm × 3 mm bone blocks from inferior extremity of pig femur along bone trabecula. The bone blocks were immersed in 20% H_2O_2 for 48 hours, 5 mmol / L NaN_3 for 12 hours, 1 mol/L NaOH solution containing 1% Triton X-100 for 10 hours, protease for 6 hours, 1:1 methanol / chloroform mixture for 24 hours, ether for 12 hours and ethanediamine for 24 hours (in soxhlet extractor). The bone was dried below 50°C and

stored in refrigerator at -80°C for three months. Take it out, dry, package and suction gas in vacuum. Then sterilize by ^{60}Co irradiation and preserve at -4°C.

Morphology observation

Observe a deproteinized bone with naked eyes, light microscope and scanning electron microscope after spraying, respectively. Calculate porosity using image analysis system.

Amino acid analysis

Deproteinized bone and fresh cancellous bone samples were detected by Hitachi I-8800 automatic amino acid analyzer. Deproteinized bone and fresh cancellous bone were grinded into powder. Add 100 mg into 2 ml 6 mol/L HCL and put them in a airtighted container with nitrogen, then store at 102°C for 48 hours, dry out and enter the column. The temperature of separation column was 57°C and the reaction temperature was 135°C. The flow rate of eluate was 0.4 ml/min. The flow rate of ninhydrin ketamine and ninhydrin buffer was 0.35 ml/min. The duration was 50 minutes.

Determination of biomechanics

Deproteinized cancellous bone were made into blocks with the size of 30 mm×3 mm×3 mm, and other 10 blocks of fresh pig cancellous bone with the same size served as control. Specimens were put on Reger omnipotent mechanical testing instrument to conduct the vertical compression test. The beam moving speed was 2 mm/min. The load measurement accuracy was 0.01mm. The load-deformation curve was recorded. In three-point bending test, beam moving speed was 2 mm/min and the deflection was 1 mm. The bending destruction curve was recorded.

Isolated culture of marrow mesenchymal stem cells (MSCs) of goat

Firstly, 4 ml of bone marrow was drawn by transfixion pin at ilium of goat. MSCs was separated, inoculated in culture flask for 1.0×10^6 cells/cm^3 and incubated in 5% CO_2 incubator at 37°C for 48 hours. Then the culture fluid was changed every 3 or 4 days routinely. When the mutual convergence of cultured cells reached 90% of growth plate, 0.25% trypsin and 0.1% EDTA were used to scatter cells. Then secondary culture was performed at 1×10^4 cell /cm^2 for 3 to 4 generations.

Cytocompatibility observation

The cells of third generation were inoculated at 1×10^4 cells /ml in 24-well culture plate and divided into two groups. Two deproteinized bone blocks were added in each well in experimental group. The cells of the same amount were added in control group. Each group contained 4 wells. Add 5 mg/ml MTT into 4 wells of each group at 4th, 6th, 8th, 10th days. Stand for 4 hours, then add 1 ml dimethyl sulfoxide and shake for 30 minutes. Draw 1.50 ml solution to detect absorption value (OD) at the wavelength of 550 nm. Take the mean value of 4 wells.

Statistical analysis

SPSS 10.0 statistical software was used for data processing. Quantitative results were represented by $\overline{x} \pm s$. The comparision between two groups was conducted by using AVONA test. $P < 0.05$ was considered statistically significant.

RESULTS

Morphological observation of deproteinized bone

In macroscopic observation, deproteinized bone was white, slightly yellowish, hard and brittle with long strip, in which lots of honeycomb pores could be observed. Light microscopy observation showed that bone was multiporous. The pores had regular shape with little residue in pores (Fig.1). Scanning electron microscopy showed that there were original bone trabecula, trabecular space and lumen systems within bone, and a natural mesh structure was observed in deproteinized bone. The average pore diameter was (386.55 ± 25.64) μm. The porosity was (75.33 ± 2.35)% (Fig. 2).

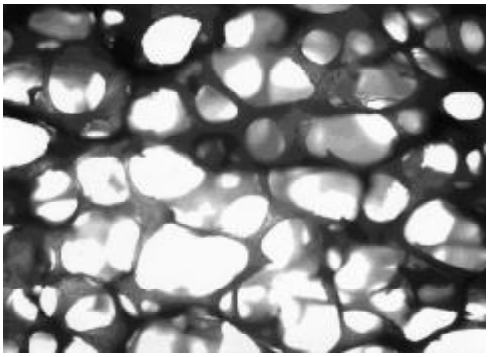

Fig.1. Deproteinized bone under light microscope (×50).

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Fig.2. Deproteinized bone under scanning electron microscope (SEM×40).

Amino acid analysis

The contents of collagenous amino acids (Gly, Arg, Lys) were relatively high in deproteinized bone. There was no significant difference as compared with fresh pig bone. The hydroxyproline accounted for 0.065% of sediments of treatment solution. The peaks of tyrosine (Tyr) and cysteine (Cys) could be observed in fresh cancellous bone, which was invisible in deproteinized bone (Figs.3. A, B).

Biomechanical detection in deproteinized bone and fresh bone

With the increase of load, cancellous deproteinized bone was highly compressed. The curve showed linear relationship. When the first peak appeared, the load was maximal. At that moment, some trabecular bone started to fracture. Afterwards, with the decline of descending compressive strength curve, the bone was compressed to some extent, then the strength increased gradually. The second peak was formed, which lasted longer. More trabecular bones were fractured and height of bone block was significantly reduced. At last, bone mass was totally destructed (Tables 1 and 2).

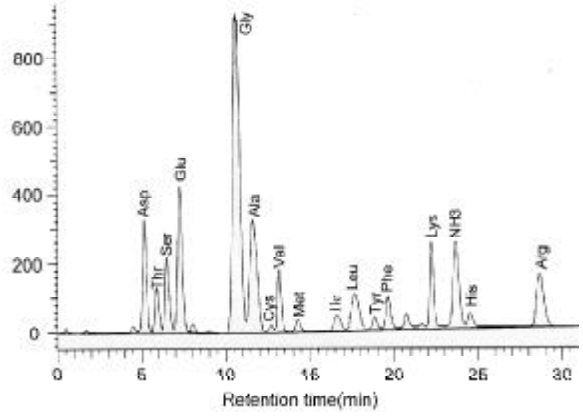

Fig.3.A. Amino acid analysis of fresh pig bone.

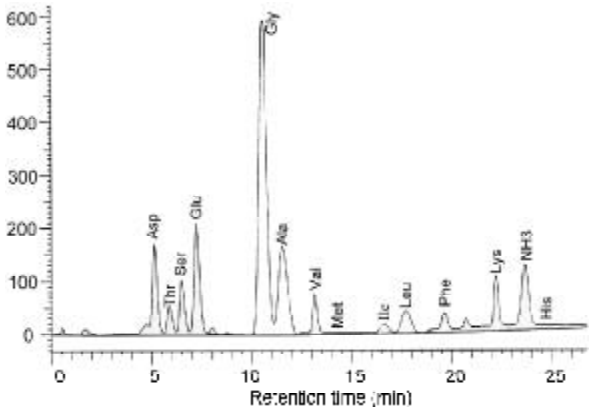

Fig.3.B. Amino acid analysis of deproteinized bone. There were no peaks of Cys, Met and Tyr.

Table 1. Comparison of fresh pig bone and deproteinized bone in compression test ($\bar{x} \pm s$)

Groups (n=20)	Sample area (mm^2)	Maximum load (N)	Compressive strength (MPa)	Yield strength (MPa)	Elastic modulus (MPa)	Fixed compressive strength (MPa)
Deproteinized bone	9.26±0.85	214.36±25.51	4.78±0.46	4.78±0.46	559.08±50.32	5.42±0.43
Fresh pig bone	9.35±0.72	195.86±18.11[1]	4.45±0.38[2]	4.45± 0.38[3]	710.80±62.74[4]	5.42±0.43[5]

P>0.05, compared with fresh pig bone group (t_1=0.722, t_2=0.998; t_3=0.863; t_4=1.256; t_5=0.687).

Table 2. Comparison of fresh pig bone and deproteinized bone in bending test ($\bar{x} \pm s$)

Groups (n=20)	Sample area (mm^2)	Maximum load (N)	Bending strength (MPa)	Breaking Strength (MPa)	Elastic modulus (MPa)	Deflection load (MPa)
Deproteinized bone	9.69±0.52	115.03±14.88	13.31±.056	14.57±0.56	0.93±.011	21.59±2.86
Fresh pig bone	9.36±0.36	128.88±13.76[1]	18.68±0.51[2]	13.42±0.38[3]	1.48±0.24[4]	16.40±1.37[5]

P>0.05, compared with fresh pig bone group (t_1=1.342, t_2=2.124; t_3=1. 682; t_4=0.743; t_5=2.236).

Observation of MSCs and the reunion of MSCs and deproteinized bone

The newly-inoculated primary cells were round with different volumes, whose nuclei were unrecognizable. On the 2th day after inoculation, some cells adhered to cell wall and became oval. On the 5th day, spindle attached cells were scattered around in changing culture fluid. On the 10th day, there was patchy integration of cells, which had covered the culture bottles (Fig.4). The complex of MSCs and deproteinized bone were cultured for 24 hours and observed by light microscope. It was found that cells could adhere and scatter on the surface or among pores. As time went by, the cells was further grown, differentiated and proliferated. Three days after co-culture of MSCs and materials, it was found under inverted phase contrast microscope that the cells and materials were closely attached to each other, gathered, and reticulated (Fig.5). Scanning electron microscope examination observed that cells were spherical, spindle clustering and located on the surface and mesh of deproteinized bone and adhered closely to deproteinized bone (Fig.6). MTT assay showed that the OD value in the same group increased as time went by and the difference between two groups was not statistically significant at the same time point (P>0.05, Table 3).

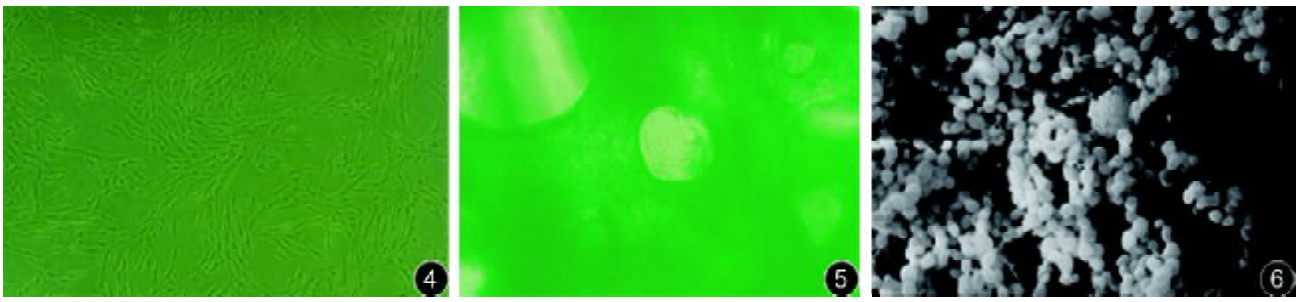

Fig.4. Marrow mesenchymal stem cells on the 10th day of primary culture under inverted microscope (×40). **Fig.5.** MSCs and deproteinized bone after 3 days of co-culture under inverted phase contrast microscope (×40). **Fig.6.** MSCs and deproteinized bone after 3 days of co-culture under scanning electron microscope (SEM×2000).

Table 3. Results of MTT assay in two groups at different time points

Groups (n=8)	4th day	6th day	8th day	10th day
Deproteinized bone group	0.985±0.007	0.257±0.011	0.258±0.018	0.250±0.026
Single cells group	0.970±0.017[1]	0.248±0.038[2]	0.250±0.035[3]	0.243±0.028[4]

P>0.05, compared with single cell group (t_1=0.655; t_2=0.798; t_3=0.863; t_4=1.032).

DISCUSSION

Large bone defect is a major problem in orthopedics. Heterogenous bone has rich sources, cheap price and the natural mesh porosity systems.[4] Moreover, its bone mineral is hydroxyapatite, which is similar to human bone. So the development of heterogenous bone is very promising. However, because of antigen difference between species, the histocompatibility is poor. If heterogenous bone is directly used for bone repair, it may cause strong immune rejection. So the removal of antigen material is necessary for heterogenous bone to be used as scaffold material. The antigenicity is mainly expressed in bone cells and endothelial cell membrane of Harver's canal.[5]

We used methanol, chloroform and hydrogen peroxide to prepare deproteinized bone. The distilled water washing was performed to get rid of harmful chemical substances. Deproteinized bone was stored at -80°C in deep freeze refrigerator for three months. After air exhaust at -700 mmHg vacuum and ^{60}Co irradiation, it was preserved at -4°C. After many rounds of physical and chemical processes, the immunogenicity of deproteinized bone was reduced by physical and chemical treatments. Long-term ultra-low-temperature freezing and radiation could decrease the antigenicity of heterogenous bone and reduce bone allograft rejection after transplantation. Irradiation could also do secondary sterilization. The detection showed deproteinized bone remained feasible properties except the reduced immunogenicity.[6] The natural lumen system was pre-

served to facilitate the entry of cell suspension during construction of bones, the transportation of nutrients and waste after implantation *in vivo*, and angiogenesis of small vessels. According to reports in literature, the sizes of deproteinized bone are different because the different animals are used in experiments.[7] Deproteinized bone is processed into 30 mm × 3 mm × 3 mm blocks, which are suitable for the repair of long bone defect (30 mm), and small defect by shortening. Width and thickness at 3 mm can increase the surface area in the same quality condition, which is conducive to seed cell adhesion. In the course of production, freezing and drying at 50°C can evaporate water in deproteinized bone. When bone is compounded with seed cells, seed cells and water can enter into deproteinized bone because of osmotic pressure.

The results of amino acid analysis showed collagenous amino acids (Gly, Arg, Lys) had high content in deproteinized bone, which was not significantly different from fresh pig bone. The hydroxyproline accounted for 0.065% of sediments of treatment solution. The peaks of tyrosine (Tyr) and cysteine (Cys) could be observed in fresh cancellous bone, which was invisible in deproteinized bone. The result is consistent with literatures.[8] It demonstrates that deproteinized bone retains most of collagenous amino acids and removes a large amount of aromatic amino acids. On one hand, the decreased immunogenicity is good for the integration and induction of bone morphogenetic protein (BMP), blocks outward diffusion and loss of BMP and provides support for osteoblasts to grow into bones. On the other hand, high content of collagen can improve mechanical strength of deproteinized bone.[9] Mechanical test showed that elastic modulus of deproteinized bone was lower than that of fresh bone and there were no statistically significant differences on other index ($P > 0.05$), which indicated that deproteinized bone had good biomechanical strength. The observation showed good cytocompatibility of deproteinized bone.

In conclusion, heterogenous deproteinized bone is an ideal scaffold material in tissue engineering for its physicochemical properties and mechanical strength. Currently, the next step is to do animal experiments to further observe its immunogenicity and provide references for clinical application in repairing bone defects.

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